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Effects of different dietary protocols on the expression of genes coding for pro- or antioxidant enzymes in the renal cortex

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List of Abbreviations

µg	Microgram
µl	Microliter
µM	Micromol
AGE	Advanced glycosylation end products
Atox1	Antioxidant-1
BMI	Body mass index
C	Celcius
CD	Control diet
COD	Coconut oil diet
DNA	Deoxyribonucleic acid
EPO	erythropoietin
FFA	Free fatty acides
Gpx	Glutathion peroxidase
H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
ICAM-1	Intercellular adhesion molecule-1
IL-1/IL-6/IL-8	Interleukin-1/ Interleukin-6/ Interleukin-8
Kg	kilograms
MCP	Monocyte chemotactic protein
MFD	Milk fat diet
mg	Milligram
NADPH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
NOS	Nitric oxide synthases
Nox	Nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase
Nox2/Nox4	Subunits of NAD(P)H oxidase
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
P	P-Value
PCR	Polymerase chain reaction
phox	Subunit of the NAD(P)H oxidase
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule-1
WHO	World Health Organization

1 Summary

The prevalence of obesity is on the rise in many parts of the world and so obesity-related disorders such as hypertension, dyslipidemia, diabetes mellitus and atherosclerosis. These diseases are the leading causes of chronic kidney and end-stage renal diseases. The metabolic changes in obesity are associated with an increased production of reactive oxygen species (ROS) which can damage DNA, cell components and disturb cell function.

This study investigates the effects of high-fat diets on the expression of genes coding for prooxidant and antioxidant enzymes in the renal cortex of male C57BL/6 mice. Furthermore, it distinguishes between a diet high in animal fat (MFD, milk fat diet) and a diet high in plant fat (COD, coconut oil diet) and investigates if the effects of the animal-fat diet are reversible by normalizing the diet. The mice were fed for 30 weeks according to the specific dietary protocol. At the end of the treatment period, mRNA expression levels of the genes of interest were measured using real-time PCR.

This work shows that the intake of high-fat diet (high in animal fat or plant fat) is associated with increased mRNA expression levels of genes coding for prooxidant and inflammatory enzymes. Specifically, the NAD(P)H-oxidase subunit Nox4 and the intercellular adhesion molecule-1 (ICAM-1) showed markedly increased expression levels. Additionally, an upregulation of the antioxidant system, particularly of the glutathione peroxidase-1 (Gpx1), in all mice with a higher fat intake was detected. No significant difference between the diet high in animal fat and the diet high in plant fat on the mRNA expression

levels of the investigated genes was seen. The effects of the animal fat diet are not reversible by diet normalization protocol used in this work.

This study provides a possible molecular explanation for the increased oxidative stress and the increased inflammation in murine renal cortex observed after high-fat diet intake.

2 Introduction

2.1 Obesity

Obesity is a major public health problem in many parts of the world and it is a key factor in the development of various chronic diseases [1]. First known as a problem in developed countries, obesity is now dramatically on the rise in low- and middle-income countries, particularly in urban setting [2]. According to the World Health Organization (WHO), about 1.5 billion adults worldwide are overweight and of these, 500 million are obese. By 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese [1].

The most common way to define obesity is the body mass index (BMI), which is determined by weight (kilograms, kg) divided by height squared (square meters, m^2). The WHO defines a BMI equal to or greater than 25 as overweight and a BMI equal to or greater than 30 as obesity (Table 1) [3].

WHO classification	**Body mass index (kg/m^2)**
Underweight	< 18.50
Normal range	18.50 – 24.99
Overweight	≥ 25.00
Obesity	**≥ 30.00**
Obese class I	30.00 – 34.99
Obese class II	35.00 – 39.99
Obese class III	≥ 40

Table 1. WHO classification of underweight, overweight and obesity according to BMI (adapted from [3])

Other approaches to quantify obesity include anthropometry (skinfold thickness), densitometry (underwater weighing), computer tomography (CT) or magnetic resonance imaging (MRI) and electrical impedance measurements [4,5]. As these methods are either less reproducible, more expensive or complicated, they are rarely used in clinical practice. Another more accepted approach is the waist-to-hip ratio. A ratio > 0.9 in women and a ratio > 1.0 in men are abnormal. This value is highly diagnostic because excess of abdominal fat is most tightly associated with the metabolic risk factors [5,6].

Obesity is caused by an imbalance between the uptake rate and the consumption of calories [1,5]. Energy intake and metabolic rate are regulated by a complex interplay of hormonal and neuronal signals. A few rare appetite regulate disorders are known but cannot account for the endemic extent of obesity [5]. It can be interpret as the consequence of the global shift in diets towards increased intake of energy-dense foods in combination with a trend towards decreased physical activity [1].

2.1.1 Health consequences of obesity

Obesity is a major cause of preventable illness and premature death worldwide [7]. Chronic diseases, such as cardiovascular diseases, diabetes, nonalcoholic steatohepatitis, musculoskeletal disorders and some cancers, are caused by obesity [1]. Hypertension, type 2 diabetes and the nonalcoholic steatohepatitis are the most prevalent ones [8-10].

Hypertension

Hypertension is a strong risk factor for stroke, left ventricular hypertrophy, heart failure, coronary heart disease, aortic aneurysm, aortic dissection, renal failure and retinopathy [6,11].

The European Society of Cardiology (ESC) defines hypertension as a systolic blood pressure level above 140 mmHg in combination with a diastolic blood pressure level above 90 mmHg. As shown in table 2, the ESC classifies hypertension in 3 different grades [12].

The incidence of hypertension correlates with the age and with the grade of obesity [11,13]. About 50% of people older than 60 years develop hypertension, in combination with obesity even about 75%. This is supported by the fact that a reduction of 10 kg body weight leads to a 10-15 mmHg lower systolic blood pressure [11].

Hypertension is a consequence of a higher cardiac output and higher peripheral resistance [11]. Obesity influences the development of hypertension in several ways: Higher body mass leads to an increased blood volume and therefore to a higher cardiac output. Vasoconstriction caused by obesity is associated with an increased sympathetic nervous system tone. The elevated insulin levels causes an insulin-mediated salt retention which is accompanied by increased blood volume [5].

Blood pressure mmHg	**Systolic**	**Diastolic**
Optimal	< 120	< 80
Normal	< 130	< 85
High Normal	130-139	85-89
Hypertension		
Grade 1	140-159	90-99
Grade 2	160-179	100-109
Grade 3	≥ 180	≥ 110
Isolated systolic Hypertension	≥ 140	< 90

Table 2. ESC definitions and classifications of blood pressure levels (adapted from [12]).

Type 2 diabetes

In the 1970 the term “diabesity” was created because of the close relationship between obesity and type 2 diabetes mellitus [14]. About 90% of individuals which develop type 2 diabetes mellitus, have a BMI >23 [13]. Especially excess of intraabdominal fat is strongly linked to the development of insulin resistance. Different mechanisms are involved in the pathogenesis of insulin resistance in combination with obesity: Elevated insulin level in plasma induces insulin-receptor downregulation. Increased level of free fatty acids in plasma leads to an impaired insulin action. Intracellular lipid accumulation and cytokines like TNF-α, interleukin 6, adiponectin and resistin produced by adipocytes, reduce the insulin-sensitivity of cells [5,15].

The majority of cases of the type 2 diabetes develop from a constellation of metabolic abnormalities including central adipositas, hypertriglyceridemia, low high-density lipoprotein (HDL) cholesterol and hypertension [11]. These

metabolic abnormalities are summarized in the term metabolic syndrome (syndrome X, insulin resistance syndrome) [5].

Nonalcoholic steatohepatitis (NASH)

The nonalcoholic steatohepatitis is defined as a necroinflammatory disorder, ultimately leading to liver fibrosis and cirrhosis [10].

NASH is closely connected with obesity, type 2 diabetes and the metabolic syndrome [10,16]. About 10% to 24% of the population in various countries has nonalcoholic fatty liver disease. In obese persons (BMI > 30), the prevalence can be increased by 57% to 74%. About 19% of obese people will develop NASH [17].

NASH develops out of a nonalcoholic fatty liver disease (NAFLD) [10]. In a first step, an accumulation of triglycerides in hepatocytes causes NAFLD. In a second step, the infiltration of inflammatory cells like neutrophile granulocytes and monocytes results in steatohepatitis [16,18]. In contrast to NASH, NAFLD is reversible by reduction in body fat and optimal diabetes control [18]. The histology of the NASH is similar to that of alcoholic liver disease, including fatty degeneration, ballooning of hepatocytes, necroinflammation and pericellular fibrosis [10].

2.1.2 Adipose tissue, an active endocrine organ

Adipose tissue is not only a passive energy store, but also an important endocrine organ which produces various cytokines. Some of them are tumor necrosis factor alpha (TNF-α), interleukin-6 or -8 (IL-6/8), angiotensin II (ATII) and monocyte chemotactic protein-1 (MCP-1). Therefore it is a source and a target of inflammatory processes [19,20]. Some of these secreted cytokines, like MCP-1 or IL-8, are well-characterized chemoattractants or activators of monocytes. In chronic state of obesity adipose tissue is infiltrated by immune cells of the monocyte/macrophage lineage [21,22]. TNF-α which is secreted from adipocytes and also from macrophages leads to adipocyte lipolysis and is involved in the genesis of inflammation [23,24] . Additionally, high plasma TNF-α levels are associated with insulin resistance, endothelial cell dysfunction, high C-reactive protein concentration and it promotes inflammatory and fibrotic processes in the kidney [19].

Metabolic changes such as hyperglycemia, hyperlipidemia and elevated inflammatory cytokines induced by obesity, elevate metabolic and inflammatory stress (Figure 1) [25]. Key enzyme of the cellular reactive oxygen species (ROS) production is the prooxidative enzyme nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H oxidases) which is increased in the adipose tissues of obese subjects (Figure 1) [25,26]. Oxidative stress plays a major role in the pathogenesis of endothelial dysfunction, hypertension and insulin resistance [26,27].

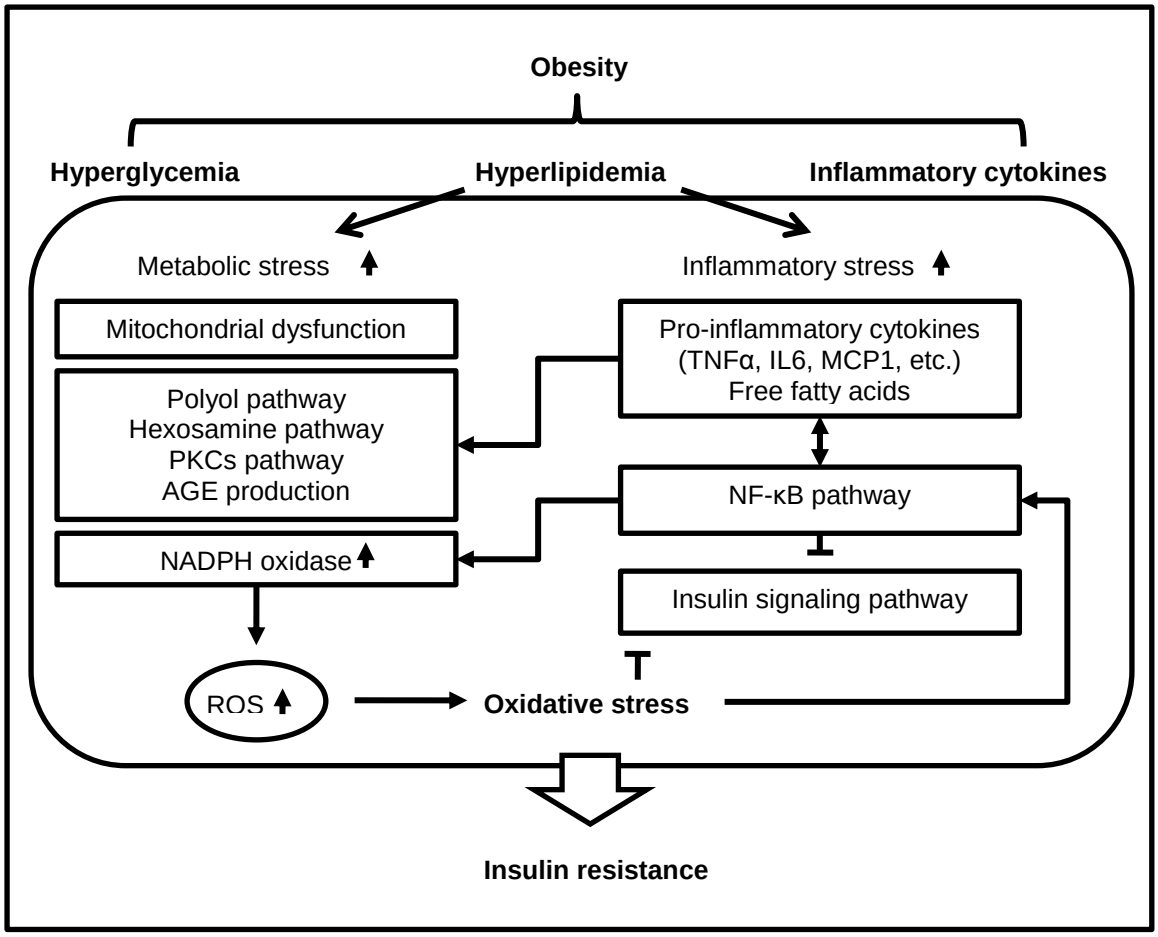


Figure 1. Metabolic changes in obesity like hyperglycemia, hyperlipidemia and inflammatory cytokines elevate metabolic and inflammatory stress. Mitochondrial dysfunction, polyol pathway, hexosamine pathway, advanced glycation end-product pathway and prooxidative enzymes NOS and NAD(P)H oxidases are involved in ROS production (adapted from [25]).

2.2 Suitability of mice as diet-induced obesity models

To understand the etiology and the effects of obesity in humans and for the development of possible treatments, animals can be used as models [28].

Because of the similar genetics to humans and genetic manipulation is readily attainable, the mouse is an established model in obesity research. Additionally, mice are small and easy to handle [28].

The male C57BL/6 mouse, also used in the present study, is the most commonly used animal model for diet-induced obesity research [29]. C57BL/6 mice show a high susceptibility to diet-induced obesity and type 2 diabetes. Fed with high-fat diet C57BL/6 mice become obese, develop mild to moderate hyperglycemia and hyperinsulinemia [29].

2.3 The kidney

2.3.1 Anatomy and function of the kidney

The paired kidneys are located in the retroperitoneal space. The kidney of an adult is approximately 10-12 cm in length, 5-6 cm wide and 4 cm thick and has a weight of about 120-300 g. Each kidney consists of a convex and a concave surface resulting in it typical bean-shaped appearance. At the renal hilum, located at the concave side of the kidney, the renal artery enters and the renal vein and the ureter leave the organ [30]. Approximately 20% of the cardiac output receives the kidney. Therefore it is a well perfused organ [31]. The kidney has a smooth surface and is surrounded by the renal capsule (lat. Capsula fibrosa) which consists of tough fibrous tissue.

Already macroscopically it is possible to differentiate the parenchyma of the kidney into two major structures: The central, deeper located renal medulla (lat. Medulla renalis) and the superficial located renal cortex (lat. Cortex renalis).

The important functional structures of the kidney are the nephrons. A nephron consists of a renal corpuscle (lat. Corpusculum renale) and the corresponding section of the renal tubule system (lat. Tubulus renalis). The renal corpuscle is located in the cortex and consists of capillary tuft (lat. Glomerulus) which is surrounded by a double-walled capsule. Each capillary tuft is built by 30 to 40 capillary-loops and is intercalated between the afferent arteriole and the efferent arteriole [30].

The kidney has an important function in the control of a variety of complex physiologic processes [32]. One important task is the control of the solute and water secretion to maintain the volume and the osmolarity of the extracellular

space [33]. It has a high filtration rate and produces about 0.7 to 1.5 l urine per 24 hours [31]. The kidney plays also an important role in the acid–base homeostasis by regulating the H^+- and HCO_3^- - secretion. Additionally, the kidney eliminates final products of the metabolism and removes drug metabolites, by conserving useful blood metabolites such as glucose or amino acids. Last but not least the endocrine function of the kidney, including the production of erythropoietin and calcitriol [32,33].

2.3.2 Effects of obesity on renal function and structure

Obesity is a risk factor for the development of chronic kidney diseases and end-stage renal disease in individuals with existing kidney disease [34].

As mentioned above (chapter 2.1.1, Health consequences of obesity) obesity increases the chance of developing hypertension and type 2 diabetes, the major causes of chronic kidney diseases. Additionally, obesity is an independent risk factor for the onset and aggravation of chronic kidney diseases. It leads to an increased renal plasma flow and hence to an increase in glomerular filtration rate. The first clinical manifestation of this glomerular hyperfiltration is an increase in urinary excretion of albumin [34]. Furthermore, obesity induces histological changes like glomerulomegaly and focal segmental glomerulosclerosis [35]. Moreover, obesity increases vulnerability to the development of renal cell cancer, in men and women [36].

2.4 Prooxidant and proinflammatory enzymes

2.4.1 Oxidative stress and role for prooxidant and inflammatory molecules in obesity

Oxidative stress is caused by imbalance between tissue oxidants like free radicals or reactive oxygen species and antioxidants. Free radicals are highly reactive molecules with unpaired electrons that easily bind with surrounding molecules. Reactive oxygen species (ROS) are oxygen-containing molecules which can have unpaired electrons and are highly reactive in tissues. On the one hand low concentrations of free radicals and ROS are necessary for normal redox cell status, cell function and intracellular signaling. On the other hand, high concentrations of ROS can damage DNA, cell components and can disturb the cell function [37].

Different mechanisms, existing in obesity, such as hyperglycemia, elevated free fatty acids in plasma and chronic low grade inflammation lead to oxidative stress [38].

Hyperglycemia

Hyperglycemia activates several oxidative pathways. In state of hyperglycemia, an increased level of advanced glycosylation end products (AGE) occurs. AGEs are the result of non-enzymatic glycosylation of protein or lipid molecules. AGEs can trigger intracellular transcription factors and increase the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Activation of these intracellular molecules leads to a higher ROS production [39]. Additionally, hyperglycemia increases the activity

of the nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H oxidase) resulting in an increased ROS production [40].

Elevated plasma level of free fatty acids

Elevated plasma level of free fatty acids (FFA) has an influence on the ROS production in different ways [38]. FFA elevate blood glucose level and produce nitroxide radicals in smooth vascular and endothelial cells. Furthermore, FFA can induce the oxidative burst in leucocytes and in this way increase the ROS production [41].

Chronic low grade inflammation

The state of chronic low grade inflammation in obesity is associated with expression of inflammatory cytokines, production of C-reactive protein (CRP) and increased level of leucocytes [38]. As described above (chapter 2.1.2, Adipose tissue, an active endocrine organ) adipose tissue produces proinflammatory cytokines like IL-6 and TNF-α. Elevated inflammatory molecules stimulate the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), and promote the migration and activation of monocytes [42]. This leads to an increased ROS production. The neutrophils, as a part of the leucocytes, elevate the ROS production via NADPH oxidase [43].

2.4.2 NAD(P)H oxidases

Nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H oxidases, (Nox)) are multi-subunit enzymes, which have been identified as a major source of reactive oxygen species (ROS) in vascular cells and in the kidney [44,45]. These enzymes catalyze the production of superoxide, which is a common progenitor of other ROS, according to the following reaction [44].

$$NAD(P)H + O_2 \rightarrow NAD(P)^+ + H^+ + O_2^-$$

The prototypical NAD(P)H oxidase Nox2 is that found in professional phagocytes such as neutrophils and monocytes/macrophages[44,46]. Figure 2 shows the components of the NAD(P)H-oxidase, which includes the membrane-integrated catalytic subunit Nox2 (also known as $gp91^{phox}$), tightly associated with $p22^{phox}$ and the regulatory cytosolic proteins $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ [46]. On stimulation, $p47^{phox}$ becomes phosphorylated and the cytosolic proteins form a complex that translocates to catalytic subunit [47]. The activation also requires participation of the small GTPase rac [46].

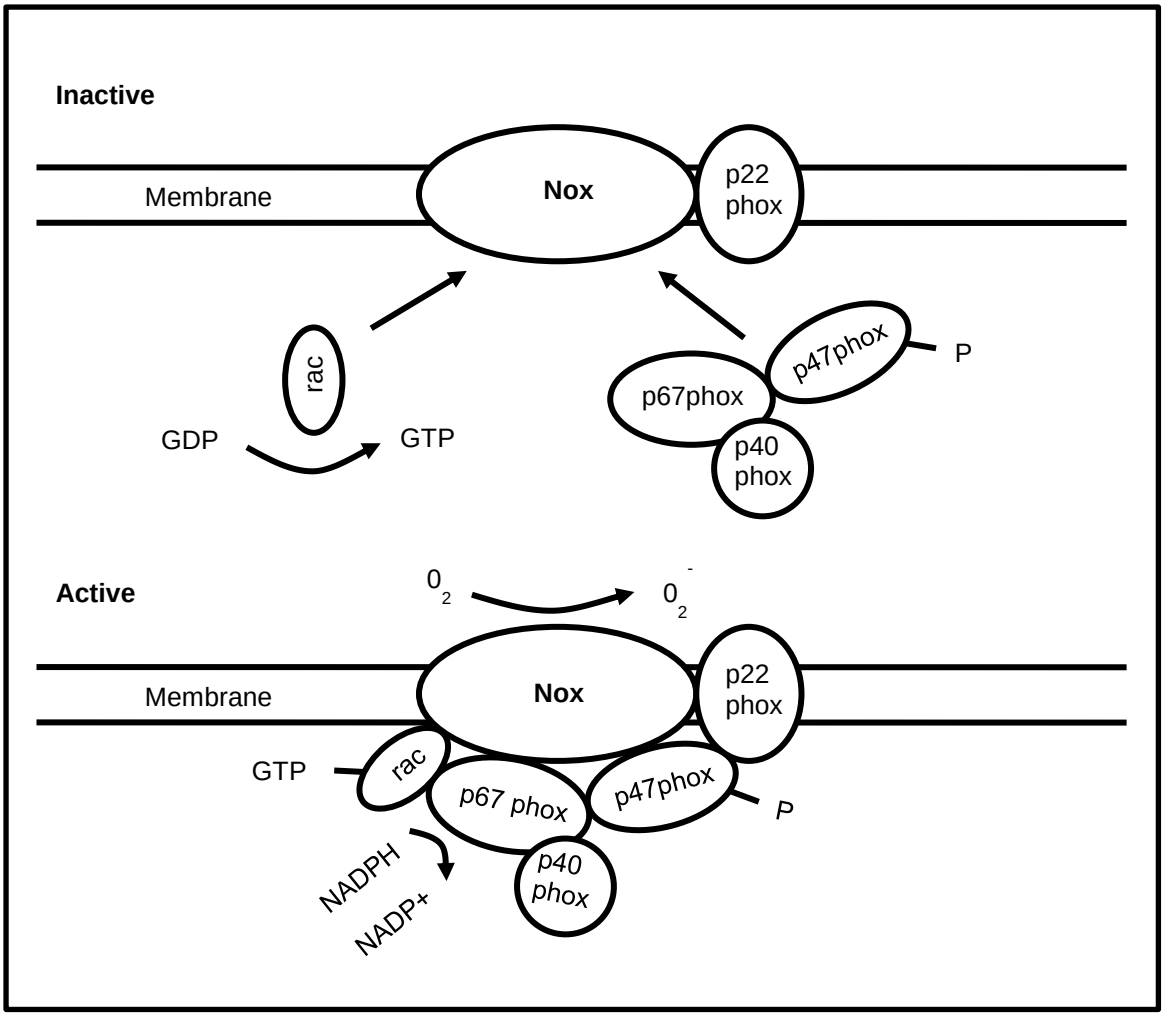


Figure 2. Structure and function of the NAD(P)H-oxidase (adapted from [48]).

Additionally to Nox2, some homologs of gp91^{phox} have been identified, termed the non-phagocytic NAD(P)H oxidase proteins. These are named Nox1 to Nox5, Duox1 and Duox2 [49,50].

The phagocytic Nox2 is dormant in resting cells and becomes activated during phagocytosis of invading microbes. Activated Nox2 releases large amounts of superoxide (oxidative burst). In contrast the non-phagocyte NAD(P)H oxidase produces continuously low levels of superoxide [45,46]. Nox2 is an important enzyme of the host defense by eliminating microorganisms through production

of ROS [51]. The importance of the enzyme is emphasized by the observation, that patients lacking functional Nox2 suffer from severe infections [51].

The non-phagocyte Nox4-enzyme (originally termed Renox) is highly expressed in the kidney, particularly in the proximal convoluted tubule cells of the renal cortex [52]. Nox4 may have a role as an oxygen sensor in the kidney and therefore a function as a regulator of the erythropoietin production [53].

The normally relatively low amount of ROS, produced by non-phagocyte NAD(P)H oxidase, is important in redox signaling in many cellular processes such as cell growth, apoptosis, migration and extracellular matrix remodeling [54,55]. For example, hormones, cytokines, oscillatory shear stress activate the non-phagocyte NAD(P)H oxidase resulting in increased ROS production[45]. As a consequence inflammation, hypertension, endothelial dysfunction and kidney failure occur [56]. In figure 3 the activation factors and the effects of the non-phagocyte NAD(P)H oxidase are summarized

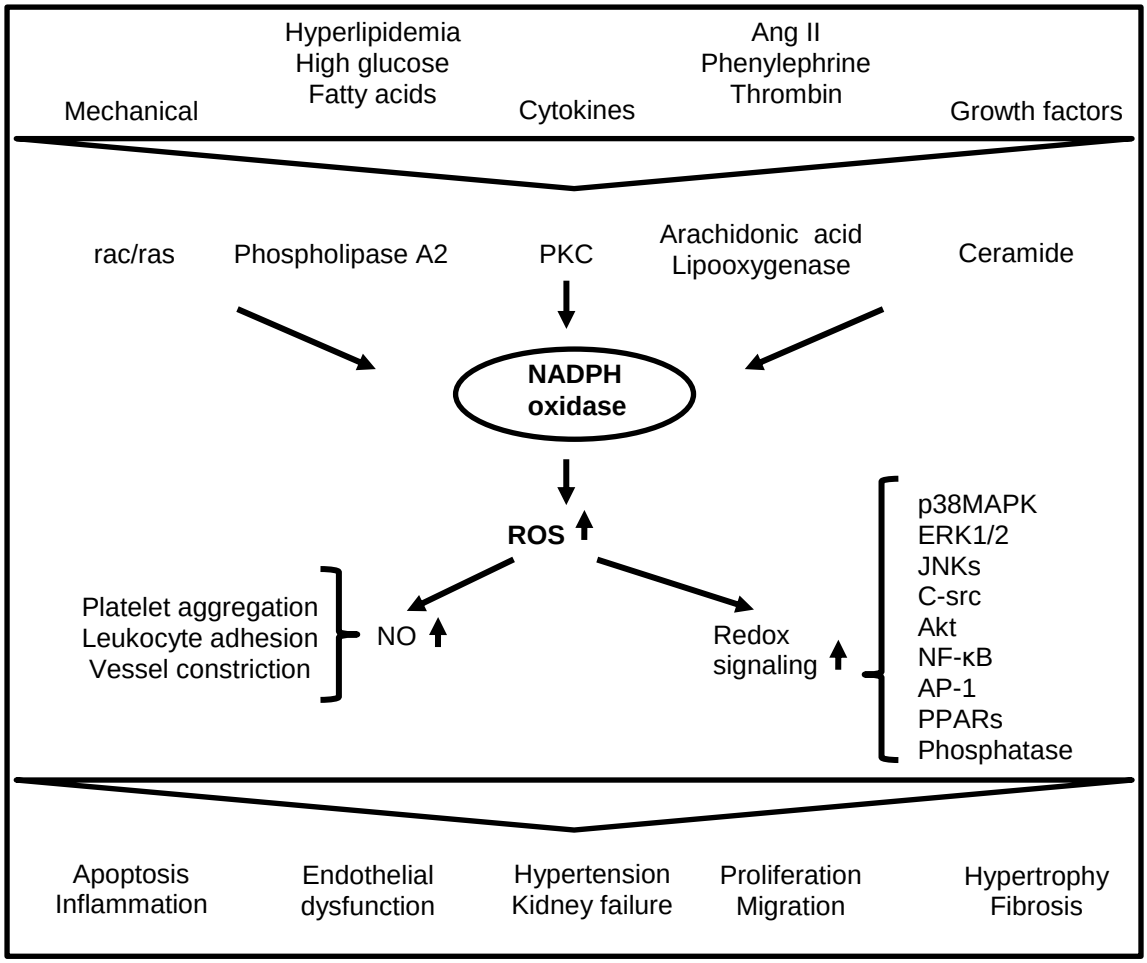


Figure 3. Activation factors and effects of non-phagocyte NAD(P)H oxidase (adapted from [56]).

2.4.3 Intercellular adhesion molecule-1

Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoprotein and a member of the immunoglobulin superfamily [57,58]. It is expressed constitutively or under conditions of inflammation in a variety of cells, including endothelial cells, parenchymal cells, hepatocytes, adipose tissue and cardiac myocites [59]. In response to inflammatory stimuli ICAM-1 works as a leukocyte adhesion receptor and mediates migration of leukocytes into tissues [60].

ICAM-1 plays a critical role in the development of diabetic nephropathy [61,62]. Hyperglycemia, advanced glycation endproducts and oxidative stress are elements of the diabetic state which induce ICAM-1 upregulation [63-65]. ICAM-1 upregulation leads to an increased accumulation of macrophages in the kidney which accelerates the development of diabetic glomerulosclerosis by producing various types of cytokines which stimulate cell proliferation and increase the expression of extracellular matrix proteins [61].

A soluble form of ICAM-1 (sICAM-1), the extracellular domain of the glycoprotein after proteolytic cleavage at the cell surface, is found in the systemic circulation [66]. The function of sICAM-1 is unclear but it is a marker of inflammation and may play a modulating role in inflammation [67].

Several studies in humans have shown that obesity, especially central adipositas, leads to elevated sICAM-1 plasma levels [68].

2.4.4 Monocyte chemotactic protein-1

Based on structural and genetic criteria the monocyte chemotactic protein-1 (MCP-1) forms together with MCP-2 and -3 a subfamily of the β-chemokines [69,70]. Chemokines are a large family of chemoattractants that direct migration of leukocytes from blood to sites of inflammation [71]. MCP-1 is a monocyte-specific chemoattractant and activator and is produced by a variety of cells on stimulation with cytokines or bacterial and viral products [69]. Very potent endogenous inducers of MCP-1, especially for mononuclear cells, fibroblasts, endothelial and epithelial cells, are the cytokines IL-1, TNF-α and interferon-γ [72,73].

A variety of inflammatory diseases, old age, cigarette smoking and obesity are correlated with higher serum MCP-1 levels [69,74]. Several studies have suggested that MCP-1 is involved in the development of atherosclerosis and higher levels of MCP-1 have been correlated with an increased risk of myocardial infarction [75,76]. Additionally, MCP-1 plays a critical role in inflammation of the kidney and it promotes the development of diabetic nephropathy [77].

2.5 Antioxidant Enzymes

2.5.1 Superoxide dismutase

Superoxide dismutases (SOD) are enzymes which catalyze the conversion of the highly reactive radical O_2^- into the more stable hydrogen peroxide (H_2O_2) in the following way [78]:

$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Three different isoforms of SOD are known: The copper- and zinc-containing homodimer SOD1 (also known as CuZn-SOD) which is localized in the cytosol, the homotetramer SOD2 (also known as Mn-SOD) which has manganese (Mn) as cofactor and is localized in mitochondria and the copper- and zinc-containing homotetramer SOD3 (also known as EC-SOD) which is localized in the extracellular space [79].

All three isoforms are expressed in the healthy kidney [80,81]. SOD1 and SOD3 are the predominant isoforms in blood vessels and play an important role in regulation of basal O_2^- levels and endothelial function [82]. The expression of SOD1 can be upregulated by several different stimuli like shear stress [83], hydrogen peroxide [84] or nitric oxide [85], whereas hypoxia may down regulate SOD1 expression in certain tissues [86]. SOD3 expression can be upregulated by cytokines [87] and other vasoactive factors like histamine, vasopressin, oxitocyin, endothelin-1, serotonin or heparin [88]. The expression of SOD2 in blood vessels is relative low, but it plays a critical role in protection against mitochondrial damage during oxidative stress [82].

2.5.2 Glutathion peroxidase

Glutathion peroxidase (Gpx) is a soluble selenoprotein, which is expressed in most tissues and is essential for defense against oxidative stress. Gpx reduces H_2O_2 and organic hydroperoxides to H_2O and corresponding alcohols, using reduced glutathione as an essential co-substrate, according to the following reaction [89]:

$$H_2O_2+2GSH \rightarrow 2H_20 + GSSG$$

So far, four different Gpx isoenzymes, Gpx1 through Gpx4, have been identified. All isoforms contain selenocystein at their active side and a selenium-dose dependent increase of Gpx1 expression has been shown [90,91].

The intracellular Gpx1 is ubiquitously expressed in humans, being particularly abundant in endothelial cells, erythrocytes, kidney and liver [90,92]. Gpx1 is a key enzyme in protecting vessels against oxidative stress and atherogenesis and different studies show that a physiological level of laminar shear stress induces an upregulation of Gpx1 expression [89]. Furthermore, decreased Gpx1 activity was observed in patients with coronary artery disease and in those with myocardial infarction [93].

The extracellular glutathione peroxidase Gpx3 is synthesized in various tissues; including heart, lung and placenta but the major source is the kidney, in particular the epithelial cells of the proximal tubules [90,92,94]. In this context, it has been hypothesized that Gpx3 may protect the proximal tubules of the kidney from localized peroxide-induced damage [90]. In contrast to Gpx1, Gpx3 has activity against phospholipid hydroperoxides, which gives Gpx3 a direct role in protection of membrane integrity [95].

2.5.3 Antioxidant-1

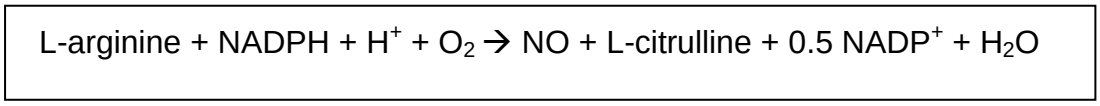
Antioxidant-1 (Atox1) is a soluble cytosolic copper carrier protein termed copper chaperons. It delivers copper to some of the secretory copper enzymes via the trans-Golgi network [96,97]. Copper (Cu) is an essential, and due to its chemical redox potential and ability to participate in free radical reactions, also potentially a toxic metal [98].

Additionally to Atox1, two other chopper chaperons have been identified: The Cu chaperone for cytochrome c oxidase (Cox1) which delivers copper to cytochrome c oxidase in the mitochondria and the Cu chaperone for superoxide dismutase (CCS) which delivers copper to SOD1 in the cytosol [99].

SOD3 as a secretory protein obtains copper trough Atox1 and for full activity of SOD3, Atox1 is required. Atox1 is not only a copper chaperon for SOD3; but is also a positive regulator for SOD3 transcription. Together, Atox1 has an important role by modulating SOD3 in the defense against oxidative stress [100].

2.5.4 Nitric oxide synthases

Nitric oxide synthases (NOS) are responsible for the synthesis of nitric oxide (NO) from L-arginine, which is described by the following equation [101,102]:



These enzymes work as homodimers and are involved in many physiological and pathological reactions [103]. Three different isoforms of nitric oxide

synthase are known: neuronal (nNOS or NOS1), originally identified as constitutive in neuronal tissue, inducible NOS (iNOS or NOS2), originally identified as being inducible by cytokines in macrophages and endothelial NOS (eNOS or NOS3), originally identified in vascular endothelial cells [104,105].

The neuronal NO synthase NOS1 is Ca^{2+}- and calmodulin-dependent and is constitutively expressed mainly in the brain but also in the peripheral neural systems, in skeletal muscle, in endothelial cells and also in the kidney where it may have a role in regulation of tubuloglomerular feedback [105-108]. Because of the expression in different tissues and the high activity in brain, NOS1 is responsible for the largest proportion of constitutive NO synthase activity [105].

The inducible NO synthase NOS2 is, in contrast to NOS1 and NOS3, Ca^{2+}- and calmodulin-independent and it is expressed in a wide range of cell types and tissues, also in the renal tubules and vasculature [105,109,110]. Inducible NOS2 is an inflammation-responsive enzyme that is upregulated in acute and chronic inflammation as part of host defense and the wound-healing process [111,112].

The endothelial NO synthase NOS3 is, like NOS1, Ca^{2+}- and calmodulin-dependent and it is constitutively expressed in endothelial cells, also in endothelial cells of renal vasculature and glomeruli and it plays a role in regulation of intrarenal vascular tone [105,113,114]. In contrast to the cytosolic NOS1 and NOS2, NOS3 is partially membrane-associated [104].

NO is a potent vasodilator and counters the effects of the renin-angiotensin, the sympathic nervous and other vasoconstrictor systems [115]. Additionally, NO reduces oxidative stress by scavenging ROS. By reducing oxidative stress, NO

inhibits the transcription of MCP-1 and VCAM-1, proteins with a central role in initiating inflammation of the vessel wall [116]. NO plays also an important role in maintaining renal function and structure and chronic NOS inhibition leads to renal injury [117].

2.6 Aim of the study

The aim of this study was to determine the effect of animal fat diet and plant fat diet on steady-state gene expression levels of genes regulating redox state and inflammation in the murine renal cortex. Specifically, the following questions were addressed:

● What are the steady-state expression levels of Nox2, Nox4, p22phox, NOS2, NOS3, ICAM-1, MCP-1, Gpx1, Gpx3, SOD1, SOD3 and Atox1 in the kidneys of control animals with normal diet?

● What is the effect of a 30 week treatment with high-fat diet of different sources (milk fat vs. coconut oil) on the renal expression of the investigated genes?

● Is the effect of a 15 week treatment with a high fat diet on expression of indicated renal genes reversible by changing the diet back to normal diet?

3 Materials and methods

3.1 Animals and diets

The study was performed with healthy male C57BL/6 mice (Charles River, Sulzfeld Germany) which were housed at the institutional animal facilities on a 12:12 h light-dark cycle. Animals had free access to food and water. All the experimental protocols and the institutional animal facilities were approved by the local authorities for animal research (Kommission für Tierversuche des Kanton Zürich, Switzerland).

At the age of four weeks, the mice were randomized and assigned to four different treatment groups, the groups CD (control diet), COD (coconut oil diet), MFD (milk fat diet) and MFD/CD. In all four groups, the mice were fed for 30 weeks according to a specific dietary protocol. In the control group, the mice were fed with the control diet (CD, Kliba Nafag 3430 Kaiseraugst Switzerland, 12.3% of total kcal from fat), the mice in the COD group were fed with the coconut oil diet (COD, Research Diets D12331, 58% of total kcal from fat) and in the MFD group, the mice were fed with the milk fat diet (MFD, Research Diets D12079B New Brunswick NJ, 41% of total kcal from fat). The MFD/CD group included mice which were fed with the milk fat diet for 15 weeks followed by control diet for 15 weeks. With these diets, the study included a vegetable fat diet (= coconut oil diet) and an animal fat diet (= milk fat diet). Table 2 shows the macronutrient composition of the three different diets.

	CD	COD	MFD
Fat	12.3	58	41
Carbohydrate	65.4	25.5	43
Protein	22.4	16.4	17

Table 2. Exact composition of the diets used (in percent of kcal).

After 30 weeks, at the end of the treatment period, all mice were fasted overnight, anesthetized (20 mg/kg body weight Xylazine, 100 mg/kg body weight Ketamine and 3.0 mg/kg body weight Acepromazine in 0.9% NaCl) and exsanguinated via cardiac puncture. Organs, which were later used to analyze RNA expression levels, were cleaned in RNA-later (Qiagen, Basel, Switzerland), snap-frozen in liquid nitrogen and stored at -80°C.

3.2 RNA extraction from the tissue

The RNA was extracted from an aliquot (not more than 30 mg) of the frozen kidney (-80°C), using the RNeasy® Mini Kit (Qiagen, CA, USA). The total RNA from the animal cells was purified according to the manufacturer's protocol and eluted in RNAse-free water.

The concentration of the RNA was determined using absorbance at 260nm and RNA solution was stored at -80°C.

3.3 Reverse-transcription

The extracted RNA was reverse transcribed using QuantiTec® reverse transcription kit (Qiagen, CA, USA) according to the manufacturer's protocol. Additional to the manufacturer's protocol, negative controls, including RNase-free water in place of the enzyme reverse transcriptase in the reverse-transcription master mix, were prepared. These controls were used to estimate contamination with genomic DNA in RNA samples.

The tubes with the cDNA and the corresponding negative controls were stored at -20°C.

3.4 Quantification of steady-state gene-expression levels using quantitative real-time polymerase chain reaction

To quantify the expression of the genes of interest, a two-step real time polymerase chain reaction (PCR) protocol was performed.

The DNA-mix of each gene was diluted 1:10 prior to adding 10 µl into the iQ™SYBER® Green Supermix (Bio-Rad Laboratories, Hercules, CA), which was aliquoted onto a 96-well PCR array plate (15 µl per well).

A Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) was used for the thermal cycling and real-time detection: Activation of the hot start Taq polymerase for 3 min (95°C), followed by 45 cycles of denaturation at 95°C for 15 sec (step 1) and annealing and extension at 60°C for 1 min (step 2).

At the end of each extension step, fluorescence was detected by using fluorescent dye which binds double-stranded DNA molecules.

The identity and specificity of amplicons were confirmed by agarose gel electrophoresis, melting curve analysis and sequencing (Microsynth, Balgach, Switzerland). Relative amounts of each mRNA were normalized to the housekeeping gene β-actin and the steady-state mRNA expression levels were calculated using the ΔCt-method and expressed as arbitrary units (AU) [118].

3.5 Agarose gel analysis

To estimate amplicon seize and for sequencing, agarose gel electrophoresis was performed using PCR-products. 2%-agarose gel was prepared by melting 2g agarose in 100ml boiling TBE buffer, adding 3µl ethidium bromide to the cooled solution and pouring it into the gel casting tray. Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows detection of DNA fragments in gels using UV light. The solidified gel was placed into an electrophoresis chamber and covered with a buffer solution. Prior to loading the DNA samples into the wells, the DNA was mixed with a loading dye. The loading dye increases the density of the DNA so it will sink into the well and it also works as a visual marker to ensure that the gel is running properly. Additionally to the DNA samples, a standard DNA sample with a DNA-ladder was loaded into one well. After all DNA samples have been loaded into the wells, the gel was run for 30 minutes with a constant voltage of 100 volts. The gel was now placed on an ultraviolet transilluminator to visualize the DNA and to compare the PCR amplicons with the DNA-ladder to estimate amplicon seize.

3.6 Extracting PCR products from agarose gel and sequencing

For the extraction of the PCR products from agarose gel, first the specific area of the gel, containing the DNA, was cut and placed in a 2ml eppendorf. UV light was used to locate DNA bands. Then, according to the Qiagen gel extraction protocol DNA fragments were purified from gel.

The purified DNA was send to Microsynth for sequencing.

3.7 Primer design

The primers used in the present study were designed using the software application Beacon designer® 2.06 (Premier Biosoft, Palo Alto, CA, USA). The sequences of the primers are shown in the appendix, chapter 7.2.

3.8 Calculations and Statistical analyses

For the statistical analyses and graphs, the StatView® 5.0.1 (SAS Institute Inc., North Carolina, USA) was used. Because of the small n-numbers, normal distribution was assumed. Statistical significance was assessed by an unpaired t-Test. All the results are expressed as mean ± standard error of the mean (SEM). Differences were considered statistically significant at p values < 0.05.

4 Results

4.1 Gene expression in kidneys of control animals

In all experimental mice, the steady state renal mRNA expression of the analyzed genes was detected. The comparison of the relative expression levels in renal cortex of control-diet fed animals is shown in Figure 3. For illustration purpose the representation of the expression levels was separated in 3 groups: low, high and very high expression (Figure 3). Five genes of interest, NOS2, NOS3, Nox2; MCP-1, ICAM-1 and Atox1 belong to the low expression level group. NOS2 is the gene of interest with the lowest expression level (Figure 3A). The second group, the high expression group, consists of p22phox, Nox4 and SOD3 which were expressed at similar levels (>30-fold more to low expression group) (Figure 3B). SOD1, Gpx1 and Gpx3 belong to the third group, the very high expression group. Gpx3 is the gene of interest with the highest expression level (Figure 3C).

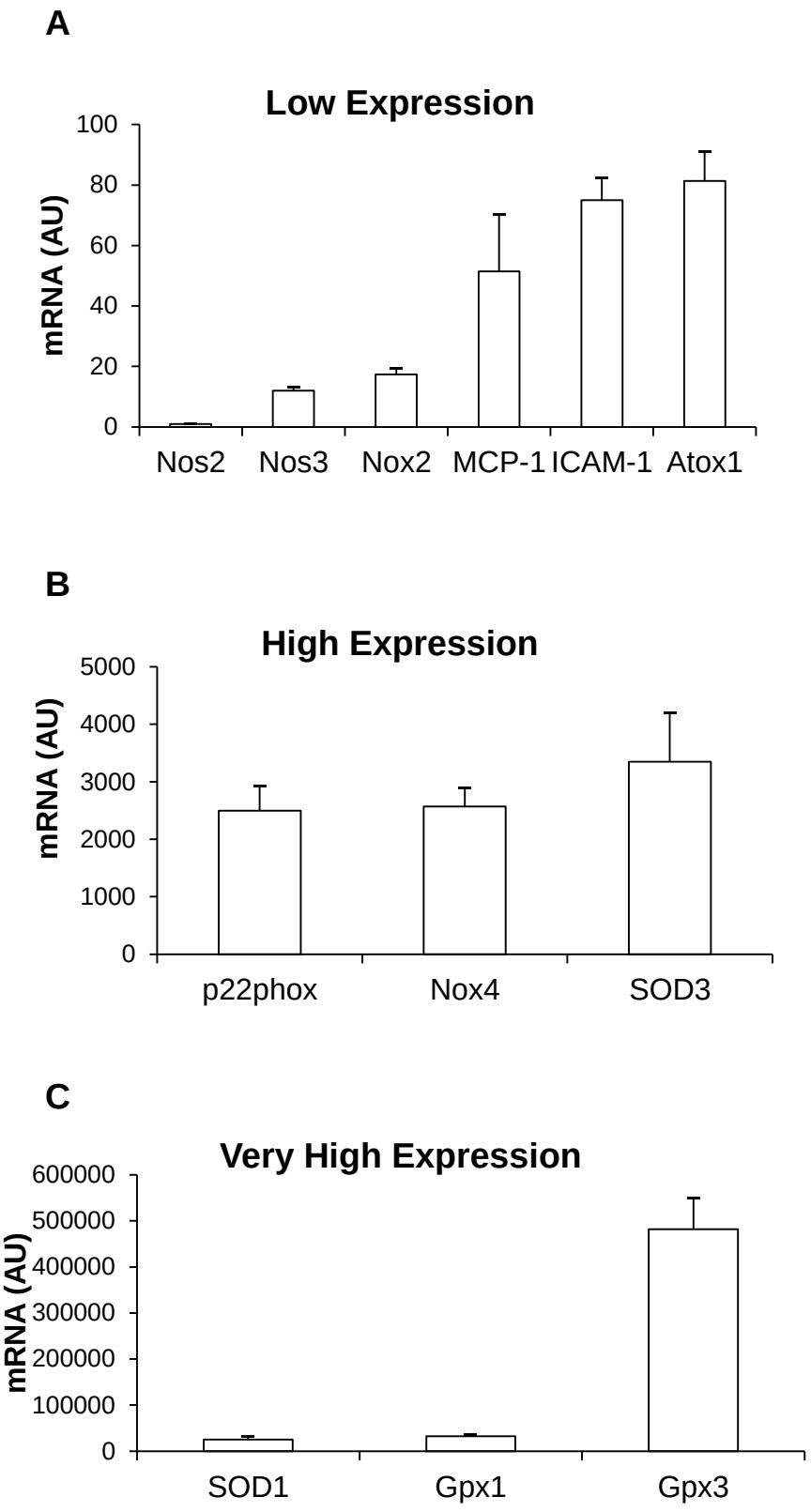


Figure 3. Steady state expression levels of renal NOS2, NOS3, Nox2, MCP-1, ICAM-1 and Atox1 (Panel A), p22phox, Nox4 and SOD3 (Panel B), SOD1, Gpx1 and Gpx3 (Panel C) in control animals. Values are mean±SEM and are expressed as arbitrary units=ΔCT of gene of interest and housekeeping gene (n=6-7). (Note different scale y-axes A-C.)

4.2 Effects of diets on renal mRNA expression levels of genes coding for prooxidant and inflammatory enzymes

4.2.1 Only Nox4 but not Nox2 and p22phox mRNA expression levels are affected by different dietary protocols

As shown in Figure 4A, the expression level of Nox4 was significantly increased in all groups with a higher fat intake compared to the control group (P<0.05 vs CD). Fat intake had no significant effect on the expression levels of Nox2 and p22phox but a trend toward a higher Nox2 expression in the mice of the MFD group compared to the control group was detected (p=0.07 vs CD) (Panel B and C).

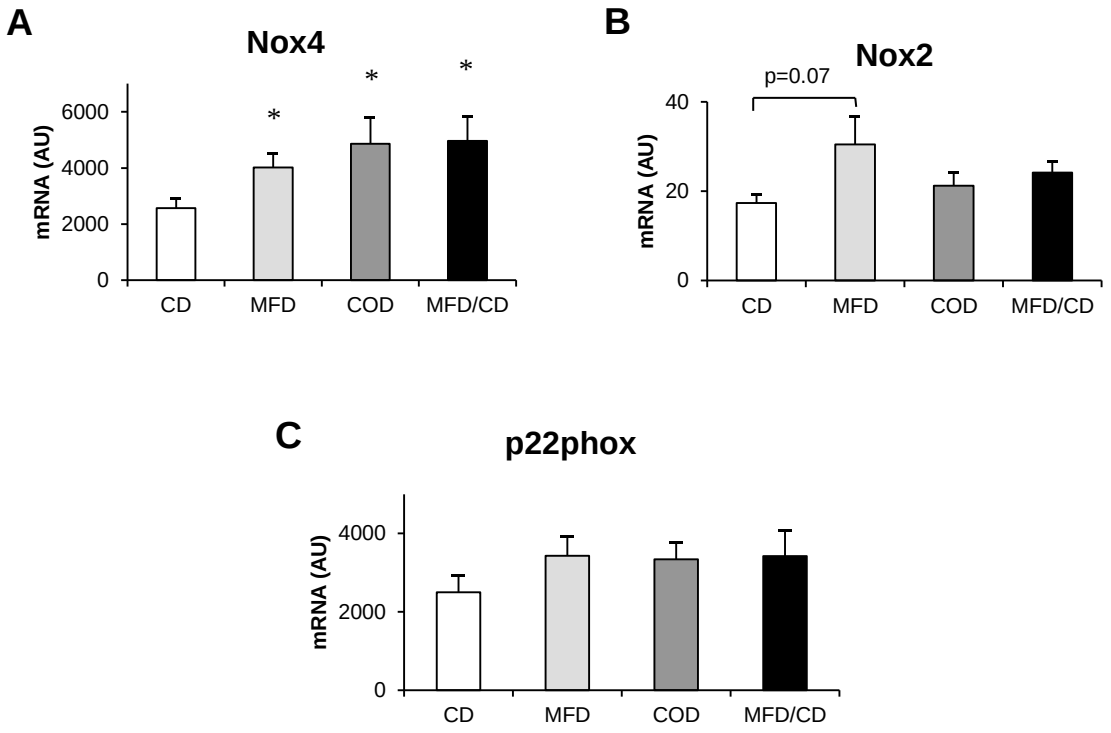


Figure 4. Effects of control diet (CD), milk fat diet (MFD), coconut oil diet (COD) and milk fat/control diet (MFD/CD) on relative renal Nox4 mRNA expression (Panel A), relative Nox2 mRNA expression (Panel B) and relative renal p22phox mRNA expression (Panel C). Values are mean±SEM and are expressed as arbitrary units=ΔCT of gene of interest and housekeeping gene (n=6-7). ***=**P<0.05 vs CD (Panel A)

4.2.2 Higher fat intake increases ICAM-1 but not MCP-1 mRNA expression levels

In all mice groups fed with high fat diets an increased renal mRNA expression of ICAM-1 was observed (P<0.05 vs CD, figure 6A). Additionally, a trend toward a lower ICAM-1 expression in the mice of the MFD/CD group compared to the MFD group was detected (P=0.11).

The increased fat intake had no significant effect on the expression of MCP-1 within the groups (Figure 6B), but a strong trend towards a higher MCP-1 expression in the MFD group was detected (P=0.051 vs CD).

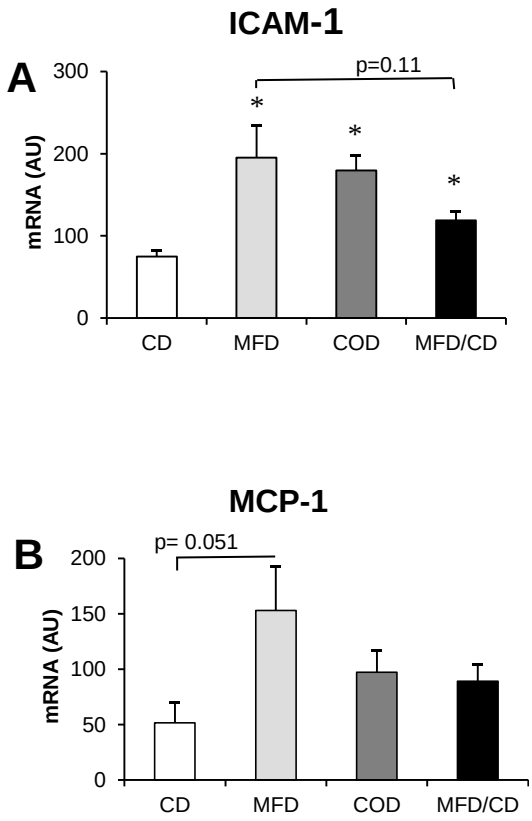


Figure 6. Effects of control diet (CD), milk fat diet (MFD), coconut oil diet (COD) and milk fat/control diet (MFD/CD) on relative renal ICAM-1 mRNA expression (Panel A) and relative MCP-1 mRNA expression (Panel B). Values are mean±SEM and are expressed as arbitrary units=ΔCT of gene of interest and housekeeping gene (n=5-7). ***=**P<0.05 vs CD (Panel A)

4.3 Effects of diets on renal gene expression levels of genes coding for antioxidant enzymes and coding for NOS

4.3.1 Gene expression of SOD1 and SOD3 are not regulated by dietary fat intake

The different dietary protocols had no effect on the expression levels of SOD1 and SOD3 in renal cortex (Figure 8A and 8B).

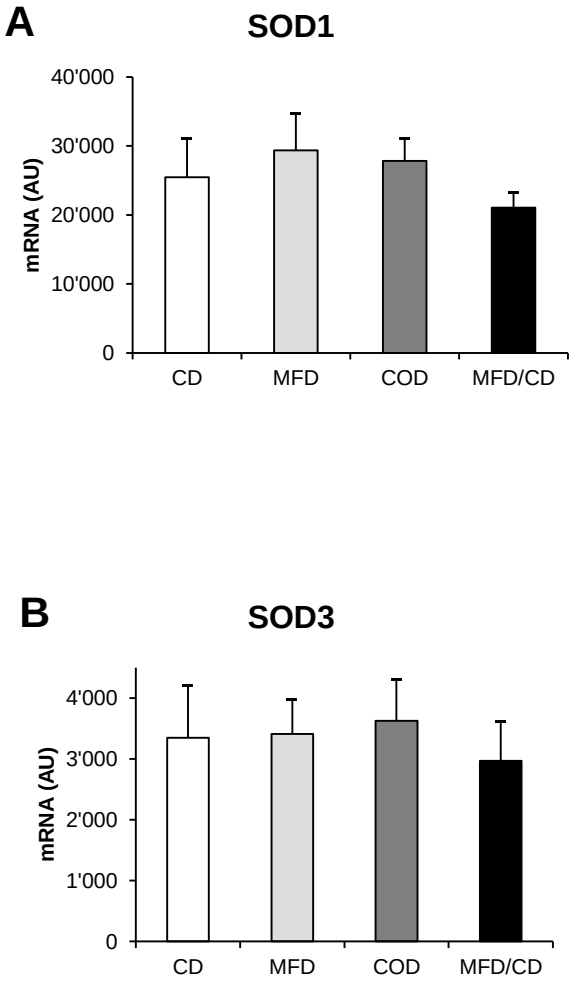


Figure 8. Effects of control diet (CD), milk fat diet (MFD), coconut oil diet (COD) and milk fat/control diet (MFD/CD) on relative renal SOD1 mRNA expression (Panel A) and relative SOD3 mRNA expression (Panel B). Values are mean±SEM and are expressed as arbitrary units=ΔCT of gene of interest and housekeeping gene (n=5-7).

4.3.2 Milk fat diet increases the expression of Gpx1 but not of Gpx3

Figure 7A shows a significantly increased expression level of Gpx1 in the MFD and the MFD/CD group (P<0.05 vs CD) and a trend to an increased Gpx1 expression level in the COD group (P=0.055). The fat intake had no significant effect on the expression levels of Gpx3 (Figure 7B).

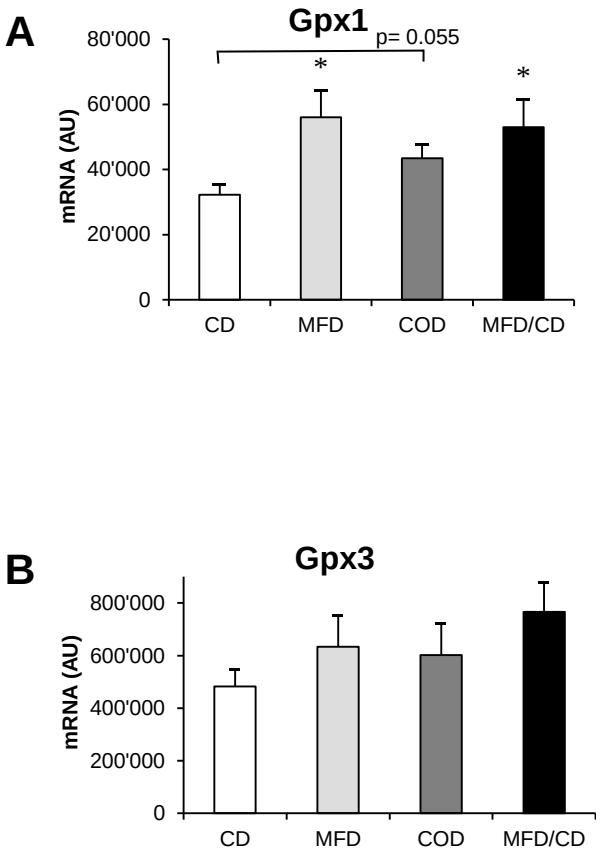


Figure 7. Effects of control diet (CD), milk fat diet (MFD), coconut oil diet (COD) and milk fat/control diet (MFD/CD) on relative renal Gpx1 mRNA expression (Panel A) and relative Gpx3 mRNA expression (Panel B). Values are mean±SEM and are expressed as arbitrary units=ΔCT of gene of interest and housekeeping gene (n=5-7). ***=**P<0.05 vs CD (Panel A)

4.3.3 Coconut oil diet increases renal gene expression of Atox1

The expression level of the copper chaperone Atox1 was significantly increased in renal cortex of COD group (P<0.05 vs CD), shown in Figure 9.

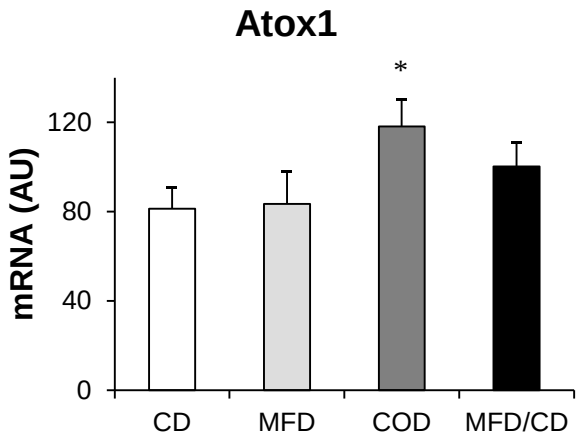


Figure 9. Effects of control diet (CD), milk fat diet (MFD), coconut oil diet (COD) and milk fat/control diet (MFD/CD) on relative renal Atox1 mRNA expression. Values are mean±SEM and are expressed as arbitrary units=ΔCT of gene of interest and housekeeping gene (n=6-7). *=P<0.05 vs CD

4.3.4 Strong trend to a higher renal gene expression of NOS3 in all mice with a higher fat intake

As shown in Figure 5A, in the group fed with MFD/CD diet NOS3 mRNA was significantly higher (P<0.05 vs CD). The COD and the MFD diet had no significant effect on the expression of NOS3 but a strong trend towards a higher gene expression was detected (P=0.06 and P=0.08 vs CD). The fat intake had no significant effect on the expression level of NOS2 (Figure 5B).

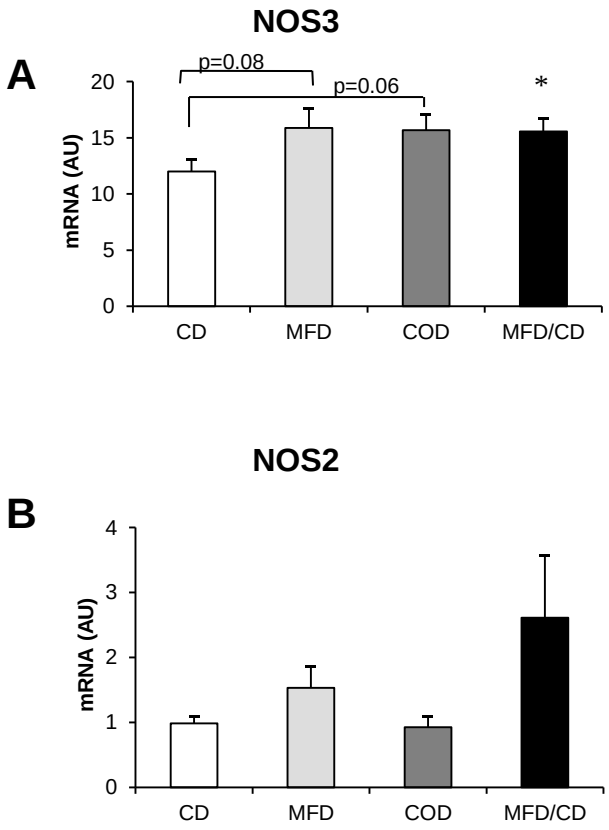


Figure 5. Effects of control diet (CD), milk fat diet (MFD), coconut oil diet (COD) and milk fat/control diet (MFD/CD) on relative renal NOS3 mRNA expression (Panel A) and relative NOS2 mRNA expression (Panel B). Values are mean±SEM and are expressed as arbitrary units=ΔCT of gene of interest and housekeeping gene (n=6-7). ***=**P<0.05 vs CD (Panel A)

5 Discussion

The present study shows that high-fat diet intake is associated with increased mRNA expression levels of genes coding for prooxidant and inflammatory enzymes (NAD(P)H oxidases, ICAM-1 and MCP-1) in the renal cortex of C57BL/6 mice. Furthermore, a trend to an upregulation of the antioxidant system in mice fed with high-fat diet was detected. There was no difference between the diet high in animal fat and the diet high in plant fat on the expression level of genes coding for prooxidant or antioxidant enzymes. A trend toward a reduction of the ICAM-1 expression after caloric restriction suggests that high fat diet induced oxidative stress may be reversible.

5.1 High-fat diet induced oxidative stress in the kidney: Expression of renal NAD(P)H oxidases, ICAM-1 and MCP-1 in mice fed with high fat diet

Oxidative stress and inflammation play an important role in the obesity related renal injury [119]. It has been demonstrated, that high fat diet induces histological changes in kidney such as mesangial space expansion in the glomeruli, collagen deposits, fibrosis, interstitial scarring and thickening basal membranes [120]. Fibrosis and scarring are dependent of numerous factors such as growth factors, cytokines and oxidative stress [35,120]. Therefore, the histological changes can be linked to the low grade inflammation existing in state of obesity [38] and the elevated oxidative stress and inflammation in kidney after high-fat diet [119,120]. Additionally, oxidative stress plays an

important role in renal damage associated with cardiovascular diseases through increased vascular inflammation and it has been demonstrated that oxidative stress activates the inflammatory cytokine NF-κB [121]. NF-κB activates the transcription of downstream inflammatory cytokines and chemokines which leads to infiltration of monocytes into tissue. These monocytes differentiate into macrophages and release additional chemokines which perpetuates the inflammation [121,122].

In this study, as indicator for oxidative stress and inflammation, the mRNA expression levels of the NAD(P)H oxidase subunits Nox2, Nox4 and p22phox as well as the mRNA expression levels of ICAM-1 and MCP-1 were measured.

NAD(P)H oxidases

Several studies showed that the multi-subunit enzyme NAD(P)H oxidase is the predominant source of ROS production in renal cortex [44,45,123]. Various NAD(P)H oxidase subunits are expressed in blood vessels, interstitial cells, glomeruli and tubule of the kidney [123]. The non-phagocyte Nox4-enzyme, a homolog of the prototypical NAD(P)H oxidase Nox2, is the predominant Nox isoform expressed in the renal cortex [52,123].

The non-phagocytic NAD(P) oxidase Nox4 is structurally related to Nox2 but there exist some functional differences between those two oxidases [56] : Nox4 generates constitutively low levels of O_2^- which are much lower than the O_2^- production of Nox2, even after stimulation [56]. A substantial proportion of ROS produced by Nox4 seems to be intracellular while ROS produced by Nox2 occur in the extracellular space [56]. An upregulation of the Nox4 level leads to an

increased ROS production resulting in morphological changes of the kidney, including glomerular hypertrophy, fibrosis and interstitial scarring [124].

Nox4, abundant expressed in distal tubular cells, has the additional function as an oxygen sensor and an influence on the expression of erythropoietin (EPO) [52]. EPO is the primary regulator of the erythropoiesis and stimulates the production of red blood cells [125]. Superoxide and its derivative ROS, produced by Nox4, destabilize HIF-1α, a dominant transcriptional activator of the gene for EPO. This inactivation of HIF-1α leads to a decreased EPO expression [52,126].

In a healthy condition, superoxide and its derivative ROS are formed by Nox4 proportional to local oxygen concentrations. Hence, a high oxygen concentration leads to a reduction of EPO expression [52].

The present study shows a significant upregulation of Nox4 mRNA level in renal cortex of mice fed with high fat diet. This finding is supported by a study of Ruggiero et al. which demonstrated an increase of Nox4 expression in the kidney of mice fed with high fat diet for 12 or 16 weeks [120].

As mentioned above, an increased expression of Nox4 and the associated elevated ROS production leads to a diminished EPO production. Since EPO is essential for a sufficient erythropoiesis, it can be hypothesized that an upregulation of Nox4 over an extended time span, as it exists in state of obesity, could cause an anemia.

Mice treated with high fat diet showed a trend to an upregulated Nox2 level. As mentioned above, there are several differences between Nox2 and Nox4: Nox2 has a stronger influence on the extracellular ROS level especially on ROS

peaks. This may suggest that an even a small upregulated Nox2 level has already a high impact on the proinflammatory state and creates more structural damage of the kidney during obesity.

On the third tested prooxidant, p22phox, the high fat diets had no significant effect. This result is comparable to the study of Simo et al. which found no significant difference in p22phox expression between age-matched groups of normotensive Wistar Kyoto rats and spontaneously hypertensive rats even though the Nox4 expression was elevated in the hypertensive rats [127]. p22phox is not only a subunit of the NAD(P)-oxidase, it is also a subunit of cytochrom b which is a component of the respiratory chain in the mitochondrion [128]. An elevation of the p22phox unit specifically associated with the NAD(P)H-oxidase in mice fed with high fat diet cannot be excluded as overall expression levels were detected and possible changes in association with different complexes remain unclear.

ICAM-1 and MCP-1

ICAM-1 as well as MCP-1 mediate transmigration and chemotaxis of monocytes and macrophages into sites of inflammation [59]. ICAM-1 and MCP-1 levels are upregulated in inflammatory conditions by several cytokines such as TNF or Il-6 [59,129,130]. These cytokines are among others produced by adipose tissue, which exists in a great measure in a state of obesity [19,20]. But not only cytokines, also ROS, especially hydrogen peroxide (H_2O_2) induces ICAM-1 expression [131]. It is known that upregulation of ICAM-1 in the kidney of mice is associated with infiltration of leukocytes (mainly macrophages), mesangial

matrix expansion and accumulation of type IV collagen. These promote glomerulosclerosis [132].

The present study shows a significant elevation of the renal ICAM-1 mRNA level in all mice with a higher fat intake. This could be a consequence of the increased Nox4 expression in the obese kidney. Additionally, a trend toward a normalization of the ICAM-1 level after changing the milk fat diet (animal fat) to normal diet is observed. The high fat diets had no significant effect to the MCP-1 expression level but a trend to an increased MCP-1 expression in the group of mice fed with milk fat diet was detected.

Taking together, the trends of Nox2 and MCP-1 suggests that a high-fat diet based on milk fat may pose a greater risk for impairment of renal function and morphological renal changes than a high-fat diet based on coconut oil.

5.2 Renal expression levels of antioxidant enzymes and NOS after high fat diet feeding

Glutathione peroxidases

The intracellular glutathione peroxidase Gpx1 is ubiquitously expressed in humans and plays an essential role in the defense against oxidative stress [90]. Gpx1 reduces organic hydroperoxides and can protect biomembranes against spontaneous lipid peroxidation which is likely mediated by H_2O_2 [133]. In healthy kidney, Gpx1 is the major isoform of the glutathione peroxidases and is responsible for 96% of Gpx-activity in kidney. Therefore, Gpx1 plays a central role in the ability of the kidney to cope with oxidative stress [134].

The present study demonstrates a significant increase of Gpx1 expression following a diet high in milk fat. These results stay in line with the study of Fujita et al. which demonstrated an upregulation of Gpx1 expression in the kidney of obese diabetic KKAy mice [135].

Since Gpx1 is the dominant antioxidant enzyme in the kidney, the significant upregulation in all mice with a diet high fat agrees with the expectation that an increased Gpx1 level acts as a compensatory mechanism to keep ROS levels under control. In mice fed with coconut oil diet, it was not possible to detect a significant effect but a trend to a higher Gpx1 level is demonstrated. The significant upregulated Gpx1 in the MFD group and the trend to a higher level of Gpx1 in the COD group suggest that Gpx1 plays a compensatory role by an upregulation of the antioxidant system. Furthermore, the non-significant upregulation of the Gpx1 in COD group supports the above formulated

hypothesis, based on the measurements of Nox2 and MCP-1, that a diet high in plant fat is less harmful than a diet high in animal fat.

SOD1, SOD3 and Atox1

In the present study the antioxidant enzymes SOD1 and SOD3 were investigated. None of the high-fat diet had an effect on the mRNA expression level of SOD1 or SOD3 in renal cortex of mice. These results are consistent with the findings of Coate et al., demonstrating that a 16 weeks treatment with high fat diet had no effect on the SOD expression in adipose tissue of male C57BL/6 mice [136]. In contrast, Roberts et al. showed a downregulation of SOD expression in the kidney of Fischer rats treated with a high fat diet [27]. The study of Furukawa et al. demonstrated a decreased expression of the antioxidant enzymes SOD in adipose tissue of obese mice, but not so in the liver and in skeletal muscles [26]. These variable results can be explained by the different animal models and different dietary protocols used in the studies. For clarification, it would be necessary to investigate the organ-specific expression of SOD in further studies.

The present study shows a significant higher expression of the copper chaperon Atox1 following the diet high in coconut oil but no change in the expression level following the milk fat diet. Atox1 is a copper chaperon for SOD3 and a positive regulator for SOD3 transcription [100]. This suggests that an upregulation of Atox1 can lead to a higher expression of SOD3. In this study, the increased expression of Atox1 in the COD group was not associated with an elevated expression of SOD3, which is not in accordance with the study of Jeney et al.

The discrepancy could be explained by the different experimental protocol used in the study: Jeney et al. compared Atox1 knockout animals to wild-type animals which showed a large difference in the Atox1 expression-level between the groups [100]. Whereas, the change in the Atox1 expression-level detected in this study may not be high enough to have an effect on the SOD3 expression.

NOS3 and NOS2

Nitric oxide synthases catalyze the nitric oxide synthesis [101]. Nitric oxide is important for the reduction of oxidative stress by scavering ROS [116]. Additionally, it plays an important role in renal physiology by regulating local arteriolar tone, tubular sodium handling and mesangial cell proliferation and hence has an anti-fibrotic effect in the kidney [107]. Endothelial NOS, NOS3, is expressed in endothelial cells of renal vasculature and glomeruli [137]. The present study shows a strong trend to a higher expression of NOS3 in the renal cortex of all mice with a higher fat intake. This effect can be interpreted as a compensatory reaction on the increased ROS production and impaired NO concentration (by scavenging ROS) which may be partly caused by the significant enhanced Nox4 expression (see above). In contrast to this result, previous studies showed no effect on the expression level of NOS3 in the kidney cortex of rats fed with moderately high-fat diet and also no effect on the expression level of vascular NOS3 of rats or mice fed with high fat diet [138-140]. Since nitric oxide produced by NOS plays an important role in protecting kidney from glomerulosclerosis [115], further investigations concerning kidney-specific nitric oxide production would be of interest.

The different dietary protocols had no effect on the expression level of the inducible NOS, the NOS2.

5.3 Limitations of the study

The body weight data of the mice were not available for the present study. Therefore, it remains unclear whether diets led to changes in weight. Histological data of the kidneys are missing which could give insight into possible structural changes in the kidneys. Measurements of oxidative stress such as ROS levels are missing. These data would provide evidence whether the change in mRNA expression levels translate to changes in renal ROS levels.

5.4 Clinical implications

The finding that ROS is linked to the development of different diseases leads to the consideration, that an improvement of the antioxidant system may prevent diseases and mortality. Additional to the enzymatic antioxidants, there are non-enzymatic antioxidants like the vitamins A, C and E, glutathione, α-lipoic acid, mixed carotenoids, coenzyme Q10, several bioflvonoids, antioxidant minerals and cofactors like folic acid, uric acid, albumin and vitamins B1, B2, B6 and B12 [141]. Many studies were conducted with the aim to show a beneficial effect on morbidity and mortality through antioxidant supplements like beta-carotene, vitamin A, vitamin C, vitamin E or selenium [141,142]. Some evidence for a protective effect of antioxidants in treating diabetes and its complications is demonstrated [141] but there is no evidence that antioxidant supplements have

a beneficial effect in primary or secondary prevention of mortality in adults [142]. Surprisingly, supplementation of beta-carotene, vitamin E and higher dose of vitamin A can even increase mortality by increasing the risk of cancer and cardiovascular diseases [142,143].

Since antioxidant supplements, including vitamins, do not display an advantageous effect and can even increase mortality, they should be considered as medical products and need to be sufficient evaluated before distributing freely to the general population.

5.5 Conclusion

The present study showed that a higher fat intake has an influence on the expression of prooxidant and antioxidant enzymes. This study could not demonstrate that these effects are reversible by diet normalization. It has to be considered that different conditions, for example a longer treatment duration, may lead to different results. Since the reversibility of the upregulation of the prooxidant system is the important factor for the success of a nutritional regime, further investigations in this direction would be of great value.

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7 Appendix

7.1 Reverse-transcription

Reverse-transcription master mix:

1µl Quantiscript Reverse Transcriptase®:	Reverse Transcriptase, also containing an RNase Inhibitor (Qiagen, Hilden, Germany)
4µl Quantiscript RT Buffer®:	Deoxynucleotid-triphosphate (Qiagen, Hilden, Germany)
1µl RT Primer Mix®:	oligo-dT and random primers (Qiagen, Hilden, Germany)

7.2 Polymerase chain reaction

PCR master mix:

12.5µl SYBR green (Qiagen, Hilden, Germany)

9.5µl RNase free water

0.5µl Primer forward (Microsynth) - (10µM in well)

0.5µl Primer reverse (Microsynth)

2µl template cDNA

PCR experiments were run on the iQ™ iCycler (Bio-Rad, Reinach, Switzerland) using specific cDNA primers (Microsynth, Balgach, Switzerland).

Primerlist:

Gene Name Accession number	Forward Primer Reverse Primer	Product size (bp)
Atox1 NM_009726	5'-GCT CTT CTC CAC AAT GCT AAC C-3' 5'-CTT AAC ACC AGT CAC ACC CTT G-3'	135
Beta-actin X03672	5'-CGT GCG TGA CAT CAA AGA GA-3' 5'-CCC AAG AAG GAA GGC TGG A-3'	180
Gpx1 NM_008160	5'-TCA GTT CGG ACA CCA GGA GAA-3' 5'-CTC ACC ATT CAC TTC GCA CTT C-3'	124
Gpx3 NM_008161	5'-CTT GGT CAT TCT GGG CTT CC-3' 5'-CCC GTT CAC ATC TCC TTT CTC-3'	148
ICAM-1 NM_010493	5'-GAC GCA GAG GAC CTT AAC AG-3' 5'-GAC GCC GCT CAG AAG AAC-3'	139
MCP-1 NM_011333	5'-GGT CCC TGT CAT GCT TCT G-3' 5'-CAT CTT GCT GGT GAA TGA GTA G-3'	121
NOS2 NM_010927	5'-GCA CCG AGA TTG GAG TTC-3' 5' AGC ACA GCC ACA TTG ATC-3'	110
NOS3 NM_008713	5'-CCT AGT CCT CGC CTC CTT C-3' 5'-ACC ACT TCC ATT CTT CGT AGC-3'	112
Nox2 U43384	5'-AAC TCC TTG GGT CAG CAG TG-3' 5'-GAG CAA CAC GCA CTG GAA C-3'	130
Nox4 NM_015760	5'-GTG AAG ATT TGC CTG GAA GAA C-3' 5'-TGA TGA CTG AGA TGA TGG TGA C-3'	148
p22phox NM_00786	5'-GTG GAC TCC CAT TGA GCC TA-3' 5'-CTC CTC TTC ACC CTC ACT CG-3'	130
SOD1 M35725	5'-TGG GTT CCA CGT CCA TCA GTA-3' 5'-ACC GTC CTT TCC AGC AGT CA-3'	151
SOD3 NM_011435	5'-TTG TTC TAC GGC TTG CTA CTG-3' 5'-CGT GTC GCC TAT CTT CTC AAC-3'	141

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